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(54) Title: REGULATION OF TYROSINE HYDROXYLASE

(57) Abstract

This invention relates to methods of regulating the effect of tyrosine hydroxylase (TH). In particular it relates to increasing the effective amount of TH in the central nervous systems (CNS) for the purpose of increasing TH–mediated dopamine production in the treatment of conditions such as Parkinson's disease.

REGULATION OF TYROSINE HYDROXYLASE

This invention relates to methods of regulating the effect of tyrosine hydroxylase (TH). In particular it relates to increasing the effective amount of TH in the central nervous systems (CNS) for the purpose of increasing TH-mediated dopamine production in the treatment of conditions such as Parkinson's disease.

BACKGROUND

Parkinson's disease is the second most prevalent neurodegenerative disorder after Alzheimer's. It is a chronic and progressive motor system disorder and is distinguished by a tremor at rest, muscular rigidity, a slowness of movement initiation and movement execution and a mask-like appearance to the face.

The cause of this disease is unknown but the symptoms are a consequence of an 80% or greater loss of the dopaminergic neurons (which produce dopamine) in the pars compacta region of the substantia nigra (SNc).

Treatments available at present only target symptoms of the disease. No drugs are currently available to intervene in the disease process. L-dopa is the most commonly employed current treatment (in order to supplement dopamine levels within the CNS), but this has limited and transient efficacy.

TH is a rate limiting enzyme for dopamine production. Upregulation of TH expression will therefore increase dopamine production in the CNS.

GPE is a tripeptide consisting of amino acids Gly-Pro-Glu. It and its dipeptide analogs Gly-Pro and Pro-Glu were first disclosed by Sara *et al* in EP 0366638. The suggestion made by Sara *et al* is that GPE has neuromodulatory properties. GPE has also been established as having neuroprotective properties and therefore having utility in the prevention or inhibition of neural cell death (WO 95/17204).

To date however, there has been no teaching or suggestion of GPE or its analogs having any direct effect on the effective amount of TH present in the CNS or being able to intervene in the Parkinson's disease process.

this is preferred. However, the administration of compounds which indirectly increase the effective amount of GPE (for example a pro-drug which, within the patient is cleaved to release GPE) is in no way excluded.

The active compound (GPE or its analog) can be administered alone, or as is preferred, as part of a pharmaceutical composition.

The composition can be administered to the patient peripherally (for example by a parenteral route such as injection into the peripheral circulation) or can be administered directly to the CNS. This latter route of administration can involve, for example, lateral cerebro-ventricular injection, focal injection or a surgically inserted shunt into the lateral cerebro-ventricle of the brain of the patient.

Conveniently, the amount of TH is increased through the administration of GPE or its analogs in the prophylaxis or therapy of Parkinson's disease.

It is also preferred that the increase of TH-mediated dopamine production is effected as part of therapy or prophylaxis of Parkinson's disease.

In a further aspect, the invention also consists in the use of GPE or an analog thereof in the manufacture of a medicament for use in increasing the amount of TH present in the CNS of a patient.

In still a further aspect, the invention consists in the use of GPE or an analog thereof in the manufacture of a medicament for use in increasing TH-mediated dopamine production for treating Parkinson's disease.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is broadly as defined above. However, those persons skilled in the art will appreciate that it is not limited only to the above but that it also includes embodiments of which the following description provides examples. A better understanding of the present invention will also be gained through reference to the accompanying drawings in which:

It is presently preferred by the applicants that GPE itself be used to increase the amount of TH/dopamine. Most conveniently, this is effected through the direct administration of GPE to the patient.

However, while this is presently preferred, there is no intention on the part of the applicants to exclude administration of other forms of GPE. By way of example, the effective amount of GPE in the CNS can be increased by administration of a prodrug form of GPE which comprises GPE and a carrier, GPE and the carrier being joined by a linkage which is susceptible to cleavage or digestion within the patient. Any suitable linkage can be employed which will be cleaved or digested to release GPE following administration.

Another option is for GPE levels to be increased through an implant which is or includes a cell line which is capable of expressing GPE in an active form within the CNS of the patient.

GPE can be directly administered as part of a medicament or pharmaceutical preparation. This can involve combination of GPE with any pharmaceutically appropriate carrier, adjuvant or excipient. The selection of the carrier, adjuvant or excipient will of course usually be dependent upon the route of administration to be employed.

The administration route can vary widely. An advantage of GPE is that it can be administered peripherally. This means that it need not be administered directly to the CNS of the patient in order to have effect in the CNS.

Any peripheral route of administration known in the art can be employed. These can include parenteral routes with injection into the peripheral circulation being a suitable example. However, alternative administration routes selected from oral, rectal, nasal, subcutaneous, inhalation, intraperitonial or intramuscular can be employed.

Two of the most convenient administration routes will be by subcutaneous injection (eg. dissolved in 0.9% sodium chloride) or orally (in a capsule).

halothane/O_2 anaesthesia. The oxygen free radical producing neurotoxin 6-hydroxydopamine (6-OHDA) which produces degeneration of dopamine neurones (8 µg/2µl) was injected into the median forebrain bundle using a 30 gauge needle (coordinates: anterior-posterior +4.7mm, right +1.6mm, vertical -8.5mm). A guide cannula was placed on the dura 7.5mm anterior from stereotaxic zero and 1.5mm from the midline on the right. The rats were left to recover at room temperature. 2 hours after the administration of 6-OHDA the rats were treated, via the guide cannula, with 3µg GPE or vehicle alone (15µl injected with a pump rate of 2µl/minute, 0.1M acetate buffer [pH6], diluted 10 times in 0.1 bovine serum albumin in 0.1M phosphate buffered saline [PBS][pH7.3]).

The rats were sacrificed using pentobarbital 14 days after 6-OHDA induced injury. Brains were perfused with normal saline and 4% paraformaldehyde and fixed in perfusion fixative overnight. The brains were paraffin embedded using a standard processing schedule. Sections (8µm) were cut through the substantia nigra using a microtome. Immunoreactivity for TH was established with sections mounted on chrome alum coated slides. Briefly, the sections were dewaxed, rehydrated and washed in 0.1M PBS. The sections were pre-treated with 1% H_2O_2 in 50% methanol for 20 minutes and then washed in 0.1M PBS (5 minutes x3). The antibodies were diluted in 1% goat serum. The sections were then incubated with rabbit (Rb) anti-TH (1:500) antibodies (the primary antibodies) for 2 days. The sections were washed using 0.1M PBS (5 minutes x 3) and then incubated with goat anti-rabbit biotinylated secondary antibodies (1:200) at room temperature overnight. The sections were washed in 0.1M PBS (5 minutes x3) and then incubated in (ExtrAvidin TM Sigma 1:200) for 3 hours and followed by H_2O_2 (0.01%) in 3,3-diaminobenzidine tetrahydrochloride (DAB, 0.05%) reaction. The sections were then dehydrated and coverslipped.

The neurons in the pars compacta region of the SNc at 3 levels in both hemispheres which showed specific immunoreactivities corresponding to TH were counted using a light microscope. The total counts of neurons were compared between the GPE and the vehicle treated group. Data were analysed with paired t-test and presented as mean ± sem. The results are presented in Figure 1.

1.5mm immediately after the injection of 6-OHDA. Either GPE (3µg/15µl) or its vehicle were infused into the right lateral ventricle 2 hours later at an infusion rate of 2µl/minute. Rats were then housed in a holding room with food and water *ad libitum* for the next 2 weeks.

The rats were then deeply anaesthetized with an overdose of pentobarbital and transcardially perfused with normal saline followed by 10% buffered formalin. The brains were removed from the skull and kept in the same fixative for the next 48 hours. A standard paraffin tissue preparation was used to process the tissue so that it could be used for immunohistochemistry. Coronal sections (8µm) were cut using a microtome, and the sections were mounted on chrome alum coated microscopy slides and air-dried. SNc sections used for immunohistochemical staining were deparaffinized, rehydrated and washed in PBS (0.1M). The sections were then pretreated with 1% H_2O_2 for 20 minutes, washed with 0.1M PBS (3 x 5 minutes) and incubated with rabbit polyclonal antisera raised against tyrosine hydroxylase (Protos Biotech, USA) diluted 1:500 with 1% goat serum for 48 hours at 4°C. The sections were washed in PBS (3 x 5 minutes) and incubated with donkey anti-rabbit biotinylated secondary antibody (1:200, Amersham, Life Science) overnight at room temperature. The sections were washed, incubated in streptavidin-biotinylated horseradish peroxidase (1:200, Amersham, Life Science) for 3 hours, washed again in PBS and then reacted in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 to produce a brown reaction product. The sections were dehydrated in a graded alcohol series, cleared in xylene and coverslipped with mounting medium.

Tissue evaluation and statistics

The number of TH positive neurons on both sides of the SNc were counted using light microscopic examination (20x magnification) at three representative levels (AP +4.2, +3.8mm and + 3.4mm) (Paxinos, *et al* (1982), New York: Academic Press). The average density from the background was also measured. The analyst was blinded to the treatment and control groups. The difference in average density between the background and TH immunostaining was calculated and used for data analysis. Right/left (R/L) ratios of both the number of TH immunopositive neurons and the average density of TH immunostaining from each level was compared between the two treatment groups using one way ANOVA. Data are presented as mean ± SEM.

made 1.3mm rostral to the rostral tip of the SNc using a retractable wire knife (David Kopf Instruments, Tujunga, CA). The knife was lowered into the brain using the following coordinates from the atlas of Paxinos and Watson (1986), Sydney: Academic Press: 3.3mm posterior to Bregma, 2.4mm lateral from midline, and 8.5mm ventral from skull, the blade was extended 2.0 mm toward midline, raised 2.5mm dorsally, retracted and extended again, and then returned 2.5mm ventrally. The wire blade was retracted and the knife withdrawn. Next, a 22-gauge metal guide cannula was permanently fixed into place supranigrally at 5.0mm posterior to Bregma, 2.0 mm lateral to midline, and 6.8 mm ventral to skull. A second set of intact unlesioned rats were cannulated supranigrally at the same coordinates.

Neurotrophic factor infusion

Animals received daily supranigral injections of trophic factors via a Hamilton syringe attached to a 28-gauge cannula 1µl of either GPE (0.3µg/µl), or 1µg of the control vehicle PBS with 0.1% bovine serum albumin (BSA) beginning immediately after lesioning and extending for two weeks post-lesioning. GPE was diluted in phosphate buffered saline (PBS) containing 0.1% BSA (pH 7.4).

Immunocytochemistry

After two weeks of treatment, animals were perfused under deep anaesthesia with PBS (pH 7.4) followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were post-fixed for 24 hours at 4°C in the same fixative then transferred sequentially to 10% and 30% sucrose in PB for 2-5 days until sunken. Floating 30µm coronal nigral sections were stained by avidin-biotin-peroxidase immunocytochemistry. Rabbit anti-rat tyrosine hydroxylase (TH) polyclonal antibody (TE101, Eugene Tech International, New Jersey, USA) was diluted 1:100 in PBS containing 0.2% Triton X-100, 3% goat serum, and 0.02% sodium azide. Sections were first incubated for 1 hour at room temperature in primary antibody vehicle. Incubation with the primary antibody was for 3-4 days at 4°C. Biotinylated anti-rabbit IgG (Vector Laboratories) secondary antibody was diluted at 4µl/ml in PBS containing 0.1% Triton X-100 and normal rabbit serum. Sections were incubated for 2 hours at room temperature, followed by an avidin-biotin-peroxidase cocktail (Vector Laboratories) incubation for 1 hour at room temperature. Peroxidase was visualized with 1 mg/ml 3,3'-diaminobenzidine in 0.03% H_2O_2 for 5 minutes. Controls were conducted by replacing the primary antibody with pre-

These findings make GPE and its analogs applicable in treating a number of neurological disorders or conditions, either therapeutically or prophylactically. Indeed, it will be apparent to those persons skilled in the art that GPE and its analogs can be employed at any time where a patient would benefit from an increase in the expression of TH/dopamine within the CNS. Neurological disorders or conditions which would benefit from this include, but are not limited to Parkinson's disease.

It will be appreciated that although the present invention is described above with reference to certain specific embodiments, the description provided is exemplary only and that the invention is not limited thereto.

dopamine production by dopaminergic neurons within the substantia nigra of the CNS by the step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

9. The use of GPE or an analog thereof in the preparation of a medicament for use in increasing the amount of tyrosine hydroxylase (TH) within the CNS of a patient for therapeutic or prophylactic purposes.

10. The use of GPE or an analog thereof in the preparation of a medicament for use in the treatment of Parkinson's disease mediated by increasing expression of tyrosine hydroxylase (TH).

11. The use of GPE or an analog thereof in the preparation of a medicament for use in increasing tyrosine hydroxylase (TH)-mediated dopamine production within the CNS of a patient.

12. The use of GPE or an analog thereof in the preparation of a medicament for use in increasing tyrosine hydroxylase (TH)-mediated dopamine production by dopaminergic neurons in the substantia nigra of the CNS in order to treat Parkinson's disease.

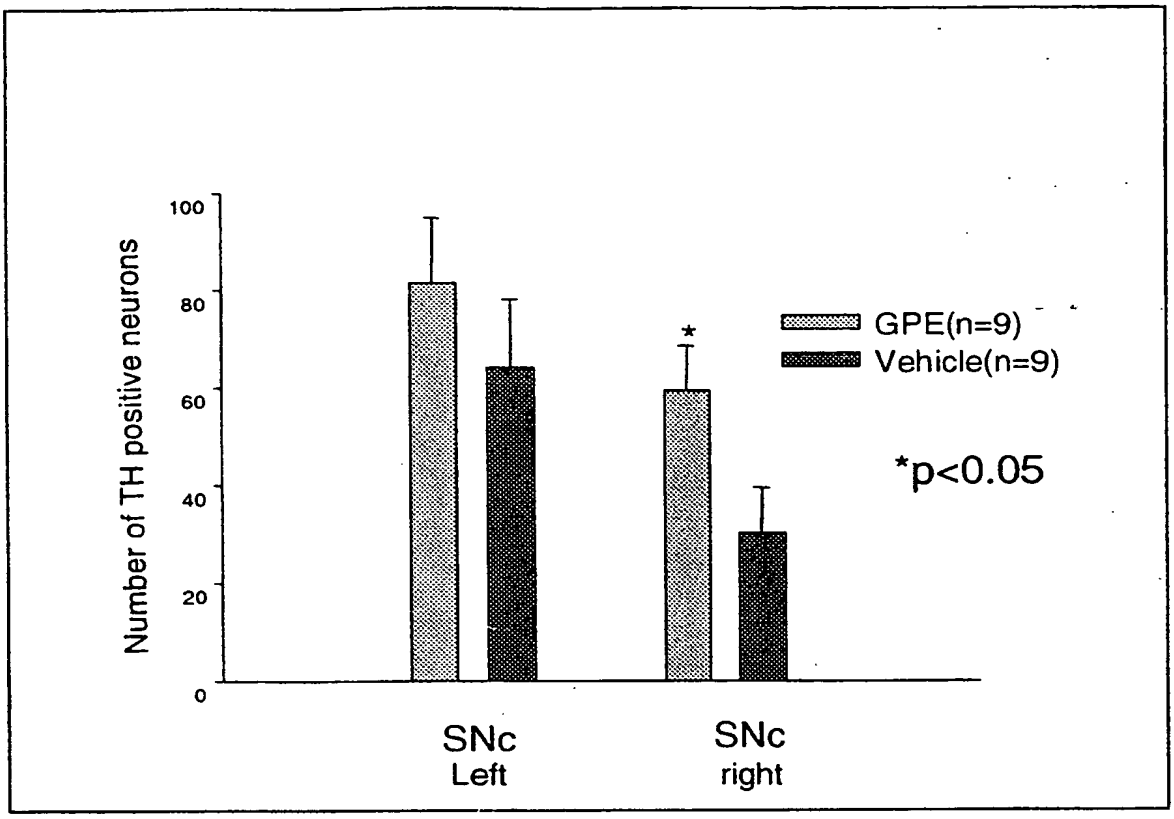
Number of TH positive neurons
100
80
60
40
20
0
*
GPE(n=9)
Vehicle(n=9)
*p<0.05
SNc
Left
SNc
right

FIGURE 2

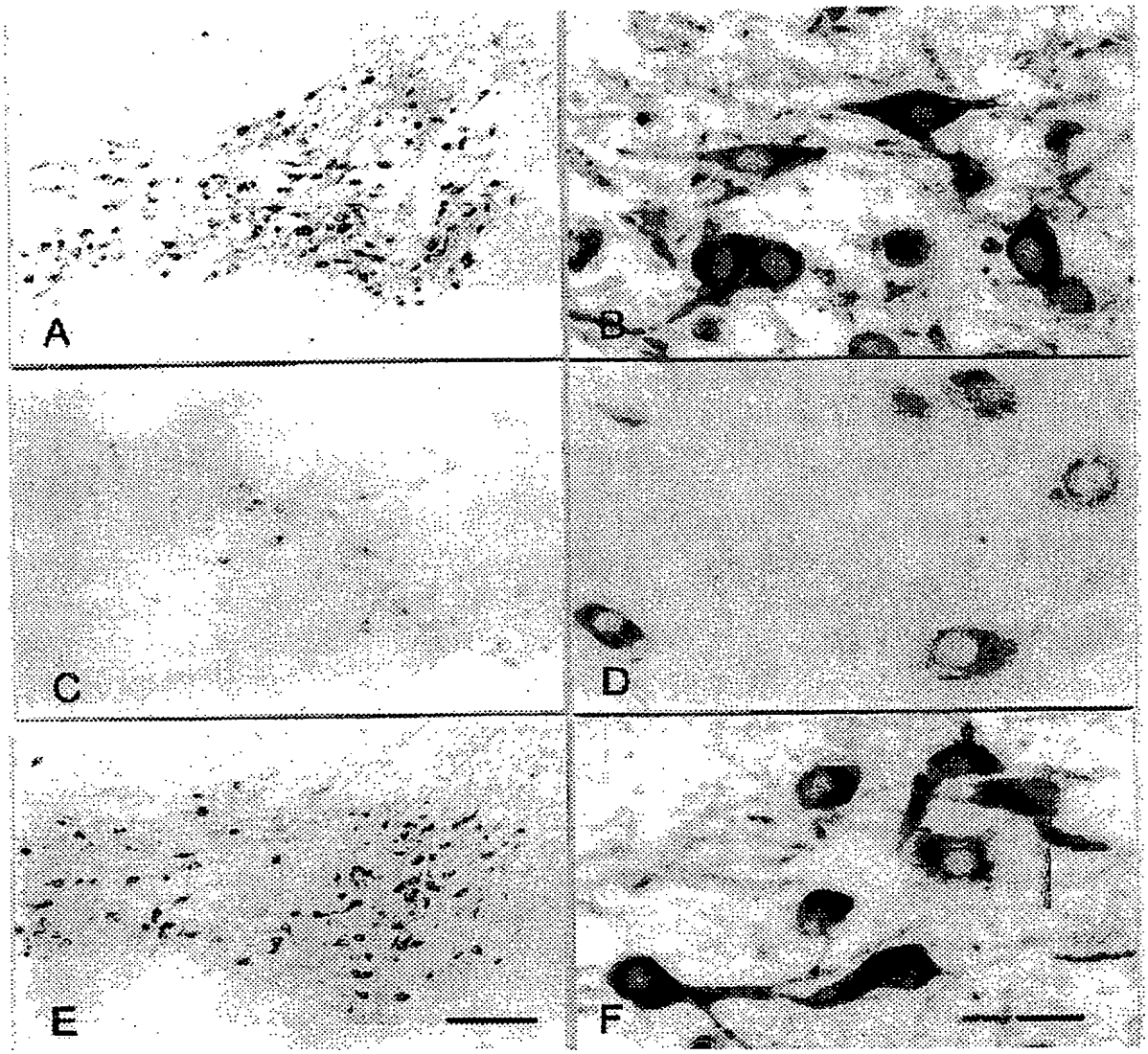

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 99/00085

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 38/06, 38/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: A61K 38/06, 38/05, 37/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: [A61K 38/06, 38/05, 37/02 and ((GPE or GLY() PRO() GLU) and (Glycine and Proline and Glutam:)]
CAPLUS: Gly-Pro-Glu, GPE and PARKINSON

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/17204 A (AUCKLAND UNISERVICES LIMITED) 29 June 1995 Whole document, page 12, line 35	1-12
A	WO 98/14202 A (AUCKLAND UNISERVICES LIMITED) 9 April 1998 Whole document	1-12
A	Vicki R Sara et al (1989) Identification of GLY-PRO-GLU (GPE), The aminoterminal tripeptide of insulin-like growth factor-1 which is truncated in brain, as a novel neuroactive peptide, Biochemical and Biophysical Research Communications, Volume 165, No: 2, pages 766-771 Whole document	1-12

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 October 1999	27 OCT 1999
Name and mailing address of the ISA/AU	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ 99/00085

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	95/17204	AU	13281/95	CA	2178711	CN	1142770
		EP	735894				
WO	98/14202	AU	46391/97	EP	929313		

END OF ANNEX